The effect of adenosine on the fluorescence responses of chlorotetracycline-loaded human polymorphonuclear leukocytes

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Chlorotetracycline has been used in human polymorphonuclear leukocytes as a probe to investigate the state of membrane-bound calcium. We examined the effect of adenosine on the fluorescence responses of CTC-loaded PMNs stimulated with the synthetic chemotactic peptide, formyl-methionyl-leucyl-phenylalanine. Adenosine inhibited the decrease in CTC fluorescence in a dose-dependent fashion and its effect was reversed by theophylline, an adenosine receptor antagonist. Removal of extracellular adenosine by incubating PMNs with adenosine deaminase abolished the effect of adenosine. These data suggest that adenosine inhibits the release of membrane-bound calcium in PMNs that normally occurs in response to chemotactic stimuli, acting via PMN surface adenosine receptors.

Adenosine; Chlorotetracycline; FMLP; Adenosine A2 receptor; Membrane-bound calcium

1. INTRODUCTION

Adenosine modulates a variety of human polymorphonuclear leukocyte (PMN) functions such as superoxide generation in response to several stimuli acting via PMN surface adenosine A2 receptors [1–6], but its mechanism of action remains unclear. Adenosine inhibits superoxide generation by PMNs in response to stimuli which cause a rise in the intracellular free calcium concentration, such as formyl-methionyl-leucyl-phenylalanine (FMLP) or the calcium ionophore A23187. However, it has little effect when PMNs are stimulated with phorbol myristate acetate (PMA) or latex beads, which activate the cells independent of a rise in intracellular free calcium [1,3]. Thus, there is a possibility that adenosine modulates PMN functions through interacting with the intracellular calcium homeostasis. However, adenosine seems to have little effect on the intracellular free calcium level in PMNs as determined by fluorescence changes of fura-2 or quin-2 [5,7], and its effect on calcium mobilization has not been elucidated.

Chlorotetracycline (CTC) is another fluorescent probe for intracellular calcium, which is considered to reflect the state of membrane-bound calcium [8–10]. The fluorescence from CTC-loaded PMNs decreases upon stimulation, and these decreases are considered to be due to the release of membrane-bound calcium [11–14]. We investigated the effect of adenosine and its analogues on fluorescence changes of CTC-loaded human PMNs stimulated with the synthetic chemotactic peptide, FMLP, to determine the role of the regulation of membrane-bound calcium in the inhibitory action of adenosine on PMN functions.

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2. MATERIALS AND METHODS

2.1. *Materials*

Chlorotetracycline, adenosine, 2-chloroadenosine, L-N^6-phenylisopropyladenosine (PIA), theophylline, hypoxanthine, inosine and FMLP were obtained from Sigma Chemical Co. 5′-*N*-ethylcarboxamideadenosine (NECA) and adenosine deaminase (ADA) from calf intestines were purchased from Boehringer Mannheim Biochemicals. Yamasa Shoyu Co. kindly provided the 2′-deoxycoformycin (dCF).

2.2. *PMN preparation*

Heparinized venous blood was obtained from healthy adult donors. PMNs were isolated by dextran sedimentation followed by centrifugation on Ficoll-Hypaque gradients [15], and contaminating erythrocytes were lysed by hypotonic saline. PMNs were washed twice and suspended in Hanks' balanced salt solution (HBSS).

2.3. *Measurement of CTC fluorescence*

CTC loading was performed according to the method of Smolen et al. [14]. In brief, PMNs were suspended at 1×10^7/ml in calcium-containing HBSS and incubated with 50 μM CTC at 37°C for 20 min. Then cells were washed once with the buffer and kept at room temperature until used. Two ml of PMN suspension in HBSS containing 5×10^6 PMNs were placed in a thermostatically controlled (37°C) cuvette in a fluorescence spectrofluorometer (Model RF-510, Shimadzu). The cells were maintained in suspension with a magnetic stirrer, underwent excitation at 380 nm, and emission signals were recorded at 560 nm.

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3. RESULTS

3.1. *Inhibition by adenosine of the decrease of CTC fluorescence in PMN stimulated with FMLP*

Fig. 1 shows typical tracings of the CTC fluorescence from PMNs stimulated with 0.1 μM FMLP with or without 1 μM adenosine. When stimulated with FMLP, the fluorescence from CTC-loaded PMNs decreased rapidly, and the maximal decrease was observed at 45–60 s after stimulation. This decrease of CTC fluorescence was inhibited when PMNs were preincubated with adenosine for 15 min at 37°C. Fig. 2 shows the dose-response data for adenosine. Adenosine inhibited the decrease of CTC fluorescence in a dose-dependent fashion and inhibition was significant at doses as low as 0.01 μM.

3.2. *Effect of theophylline*

Fig. 3 shows the effect of theophylline, a known adenosine receptor antagonist. When 10–100 μM theophylline was added together with adenosine, the inhibitory effect of 0.1 μM adenosine was reversed. These results are consistent with the hypothesis that adenosine binds with adenosine receptors to inhibit the decrease of CTC fluorescence.

3.3. *Effects of adenosine metabolites*

To determine the possible role of adenosine metabolites, we investigated the effect of an ADA inhibitor (2′-deoxycoformycin; dCF) on the changes in CTC fluorescence produced by adenosine. The effect of adenosine was slightly reversed by the addition of dCF, but this change was not statistically significant. The major metabolites of adenosine deamination, inosine and hypoxanthine, did not inhibit the decrease in CTC fluorescence even at as high a concentration as 100 μM (Table I). Furthermore, 2-chloroadenosine, a poorly metabolizable analogue of adenosine, inhibited the decrease in CTC fluorescence as potently as adenosine (data not shown). These results showed that adenosine deamination by ADA was not required for inhibition of the decrease in CTC fluorescence.

3.4. *Effect of ADA*

The results shown above indicated that the effect of adenosine on CTC fluorescence was mediated via sur-

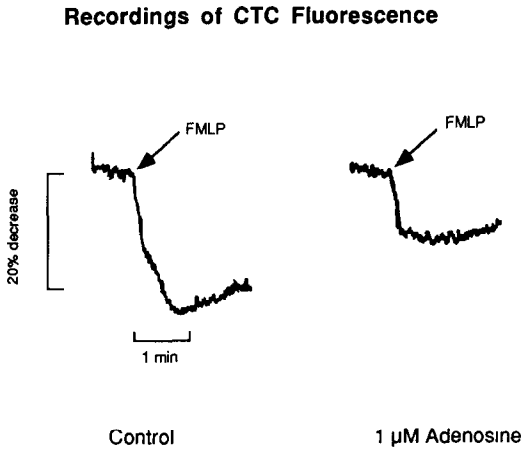

Fig. 1. Typical tracings of the changes in CTC fluorescence with or without 1 μM adenosine. PMNs were stimulated with 10^{-7} M FMLP at the indicated time. The right tracing shows the result when PMNs were preincubated with 1 μM adenosine at 37°C for 15 min before stimulation. Adenosine inhibited the decrease in fluorescence in CTC-loaded PMNs.

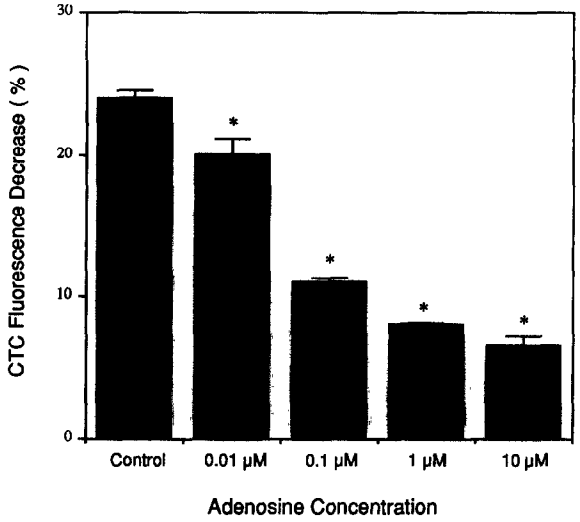

Fig. 2. The dose-response data for the effect of adenosine on the decrease in CTC fluorescence of PMNs stimulated with 10^{-7} M FMLP. PMNs were preincubated with various concentrations of adenosine at 37°C for 15 min before stimulation. Values represent the mean ± SD of three determinations. * Significantly lower than control, $P < 0.01$ by Student's t-test.

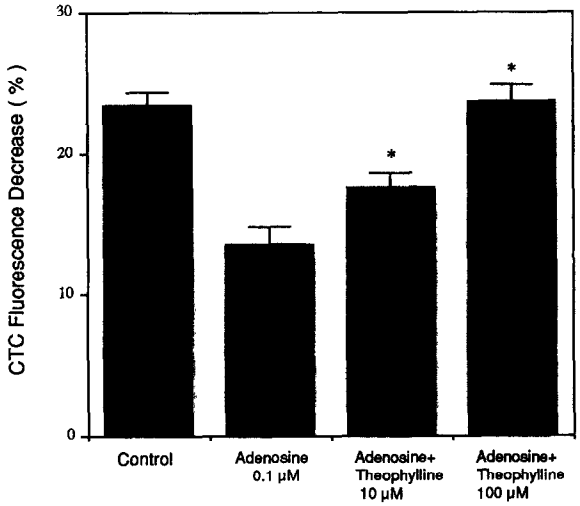

Fig. 3. The effect of theophylline, an adenosine receptor antagonist. PMNs were incubated with 0.1 μM adenosine alone or with adenosine plus 10 or 100 μM theophylline at 37°C for 15 min, and then stimulated with 10^{-7} M FMLP. Theophylline reversed the effect of adenosine. Values represent the mean ± SD from three determinations. * $P < 0.01$ vs 0.1 μM adenosine.

Table I

Treatment	Decrease in CTC fluorescence (% control)
1 μM adenosine	45.4 ± 6.6 ($P < 0.01$)*
1 μM adenosine + 5 μM dCF	52.6 ± 4.5 ($P < 0.01$)*,**
100 μM inosine	95.4 ± 3.5 (N.S.)
100 μM hypoxanthine	101.0 ± 3.1 (N.S.)

2′-Deoxycoformycin (dCF), an ADA inhibitor, was added 10 min before adenosine. dCF did not reverse the inhibitory effect of adenosine significantly. Inosine and hypoxanthine, even at high concentrations, did not inhibit the decrease in CTC fluorescence caused by stimulation of PMNs with FMLP. Values represent the mean ± SD from three determinations. *,** Significantly lower than the control (Student's t-test). ** Not significantly different from 1 μM adenosine. N.S., not significant vs control

face adenosine receptors. When extracellular adenosine was removed by incubating the PMNs with ADA (0.25 U/ml), not only was the effect of adenosine abolished but also the decrease in CTC fluorescence was augmented (Fig. 4). This augmentation was also observed when ADA was added without adenosine. These results suggested that extracellular adenosine molecules were required for adenosine to have any effect on CTC fluorescence, and that the amount of endogenously produced adenosine present in our experimental system was also sufficient to inhibit the decrease of CTC fluorescence. In fact, Cronstein et al. [1] have reported that supernatants of PMN suspensions may contain significant amounts of endogenous adenosine, and ADA treatment has been reported to augment some PMN functions [1,7] perhaps by the removal of endogenous adenosine.

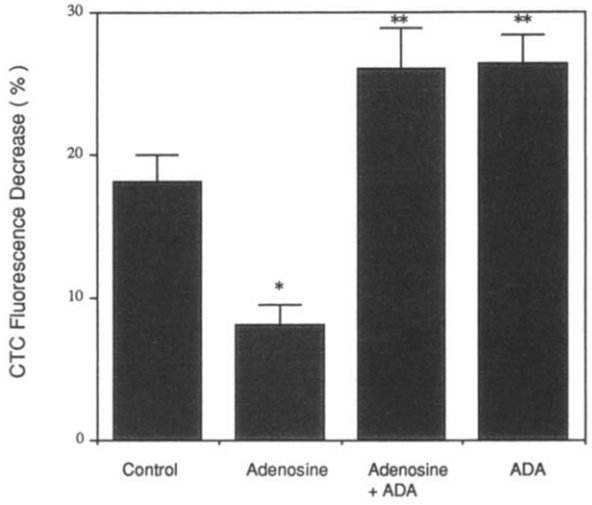

Fig. 4. PMNs were incubated with 1 μM adenosine, 1 μM adenosine plus 0.25 U/ml of ADA, or 0.25 U/ml of ADA alone at 37°C for 15 min before stimulation with 10^{-7} M FMLP. ADA treatment not only abolished the inhibitory effect of adenosine, but actually augmented the decrease in CTC fluorescence stimulated by 10^{-7} M FMLP. * $P < 0.01$, significantly lower than control; ** $P < 0.01$, significantly higher than control.

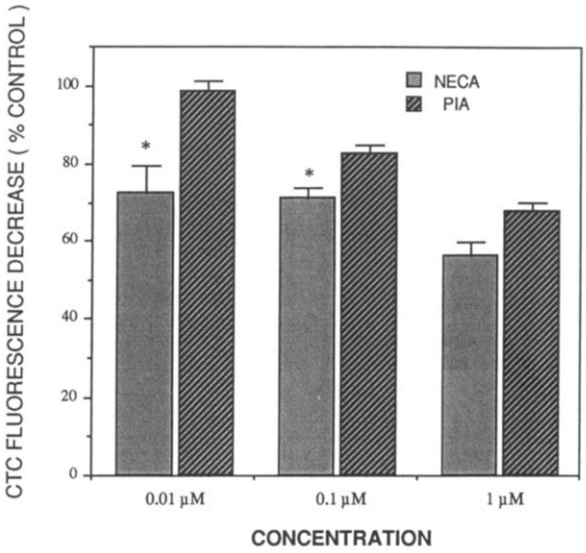

Fig. 5. The effect of NECA and PIA on the change in CTC fluorescence. Each adenosine analogue was added 15 min before stimulation at the indicated concentration and their effects on CTC fluorescence were compared. Mean ± SD of three determinations. * Significantly lower than PIA, $P < 0.05$.

3.5. *Characterization of adenosine receptors*

NECA and PIA are adenosine analogues with different affinities for adenosine A_1 and A_2 receptors. They were both effective in inhibiting the decrease in CTC fluorescence, with NECA being more effective than PIA (Fig. 5). This order of potency is characteristic for adenosine A_2 receptors, as previously reported [16,17].

4. DISCUSSION

Adenosine appears to inhibit PMN functions only in response to those stimuli which are dependent on a rise in the intracellular free calcium concentration [1,3]. Therefore, several investigators examined the effect of adenosine on calcium mobilization, but found that it seemed to have little effect on intracellular free calcium levels as determined by the fluorescence calcium indicators, quin-2 or fura-2 [5,7].

The release of membrane-bound calcium is one of the earliest events after PMN stimulation and is thought to be important in PMN signal transduction [11–14]. CTC has previously been used as a probe for membrane-bound calcium [8–10], so we utilized it to investigate the effects of adenosine and its analogues on the stimulation of PMNs by the synthetic chemoattractant, FMLP. Adenosine inhibited the decrease in fluorescence of CTC-loaded human PMNs in response to FMLP in a dose-dependent fashion. The effect of adenosine was reversed by theophylline, which is a potent adenosine receptor antagonist. Adenosine metabolites produced by ADA had little effect on the decrease in CTC fluorescence and an ADA inhibitor

could not reverse the effect of adenosine significantly. However, removal of extracellular adenosine by ADA both abolished the effect of adenosine and augmented the decrease in CTC fluorescence, indicating that extracellular adenosine is required to inhibit the change in fluorescence. Also, NECA was more potent than PIA in inhibiting the decrease in CTC fluorescence, suggesting that adenosine A_2 receptors were involved in this process.

The results shown above indicate that physiological concentrations of adenosine inhibit the release of membrane-bound calcium in PMNs stimulated with FMLP, acting via surface adenosine A_2 receptors. Therefore, it is possible that the inhibitory effects of adenosine on PMN functions may be caused by blocking the release of membrane-bound calcium that normally follows PMN stimulation.

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